The Development of Synthetic Peptides as Potential HTLV-1 Fusion Inhibitor Therapeutics

A Senior Honors Thesis

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Abstract

Human T lymphotropic virus type 1 (HTLV-1) was the first isolated retrovirus and is the causative agent of Adult T cell leukemia or Tropical Spastic Paraparesis which develops in 6-10 % of HTLV-1 positive individuals. Viral transmission takes place vertically or horizontally via cell to cell contact and syncitia formation between infected and non-infected cells. The viral envelope is responsible for the attachment and entry of the virus into the host cell. Env is composed of two subunits: Gp 46 referred to as the surface subunit, and the gp21 transmembranous subunit. It has been proposed that gp46 is mainly involved in host cell receptor binding and gp21 is involved in post binding events resulting in the fusion of host cell membrane with the infected cell membrane. There has been no effective cure against HTLV-1 and it is resistant to conventional means. Therefore investigations in possible therapeutic approaches are very important. One possible therapeutic strategy against HTLV-1 is the use of peptide fusion inhibitors to prevent transmission of the virus. Two peptide sequences, 400-429 (P400) and 197-216 (P197) from gp21 and gp46 respectively, have been shown to inhibit syncitia in *in vitro* HTLV-1 transmission studies. These investigations have been supported by a similar success with HIV. T-20, a potent viral fusion inhibitor, is analogous to P400 in that it was designed to mimic part of the transmembranous subunit, gp41 of HIV. Consequently it interacts with gp41 inhibiting viral fusion from taking place and therefore limiting viral infection. T-20 clinical trials have shown that HIV viral load declined in AIDS patients after a series of T-20 adminstration. One observed drawback of using T-20 is its short bioavailability leading to frequent dosage and consequentially resulting in the high cost of treatment. The goal of this project was to develop versions of P400 and P197 with

longer half lives using the retro-inverso peptide modification strategy. This involved the use of protease resistant D-amino acids instead of the biologically relevant L-amino acids while inverting the peptide backbone sequence to achieve the same side chain orientation as the parent peptide with a similar retained function. In this project P400 was compared with a previously described mutant of HTLV-1 ($P^{cr}400$) in order to determine the best candidate for retro-inverso strategy. Using a three cell line syncitia inhibition assay, it was found that P400 was more effective at inhibiting syncitia and was chosen for retro-inverso (RI) development. Retro-inverso P400 (RIP400) was synthesized, characterized, and evaluated with P400 to determine if similar efficacy levels would be retained. It was found that RIP400 was less effective then P400. However RIP400 displayed comparable efficacy with the second documented fusion inhibitor P197. Further investigations were conducted using cross-reactivity ELISA to explore the reasons behind the discrepancy observed. Furthermore, a peptide sequence 175-218 (a potent B cell epitope) of the gp46 region was compared with P197 for the possibility of a better alternative to use for the retro-inverso strategy. No difference was observed between both peptides. P197 was also tested in combination with P400 for a possible synergistic effect since they target different subunits, which may encourage the development of RIP197 as a combination with RIP400. The finding of this investigation revealed that no synergism was achieved. Because of the inability to find a better version than P197, the absence of an added advantage of using P197 in combination with P400, and the cost of retro-inverso peptides, the pursuit for RIP197 synthesis was not completed.

This project explored the possibility of developing potential longer half-life therapeutics against HTLV-1 through the use of retro-inverso modification strategy. It

has been shown that the retro-inverso strategy was successful with RIP400 peptide in its ability to retain partial and significant efficacy from its parent peptide. Further *in vivo* and half life experiments are necessary for confirmation about the actual efficacy of the potential therapeutics and the success of retro-inverso strategy in increasing half life of treatment. This project has important implications in HTLV-1 therapeutic development.

Introduction

Human T lymphotropic virus type 1 was the first discovered retrovirus and was classified among the type C retroviridae [1, 2, 3]. It is the etiological agent of two main diseases, adult T-cell leukemia / lymphoma (ATL), and HTLV-1 associated myelopathy / tropical spastic paraparesis (HAM/TSP). Individuals with ATL may generally fall into three categories: a) preleukemic or pre-ATL stage, where the infected individual is asymptomatic and diagnosis of the pathological condition is coincidental through serological screening or detection of lymphocytosis (lymphocytes with abnormal morphology) [3]. b) About 50 % of individuals at that stage develop into an acute stage of ATL, which is characterized by elevated leukocyte count often eosinophilia and neutrophilia as well as T cells with morphological abnormalities. Consequently the diseased individual expresses clinical conditions that are directly related to leukemic infiltration of different organs, some of which include hepatosplenomegaly, lymphadenopathy, and skin lesions. This form of ATL is highly malignant and resistant to chemotherapy. Individuals may die within 6 months after the first onset of the disease. c) Chronic/smoldering ATL is another stage that is common among 30 % of symptomatic HTLV-1 positive individuals, which is less aggressive than acute ATL and therefore may become a translational state to acute ATL. In HAM/TSP, patients develop progressive myelopathy, which is gradual damage to the CNS, particularly the spinal cord. This is caused by chronic inflammation that targets virally infected cells in the CNS. Anti HTLV-1 antibodies in the cerebrospinal fluid are therefore detected in HAM/TSP patients. HAM/TSP resembles multiple sclerosis in its clinical manifestation including

spasticity in the lower extremities, urinary and fecal incontinence as well as hyperreflexia [3].

However, most of the HTLV-1 positive individuals are asymptomatic and are considered carriers. Therefore they heavily contribute to the silent transmission of the virus. Approximately 10-20 million individuals world wide are HTLV-1 positive, of which 4 % develop one of the two pathologies described earlier [2]. Major endemic areas include Japan, Central and South America, and Africa. HTLV-1 infection has been transmitted to the United States through immigrants from endemic areas and it spreads in two main directions: Horizontal and vertical. Horizontal transmission includes sexual intercourse, intravenous drug abuse between addicts, and any type of transfusion of HTLV-1 whole blood. Vertical transmission occurs from mother to child through breast feeding. During pregnancy, HTLV-1 virions or infected cells are unable to cross the placenta and therefore is a protection for the fetus against acquiring the infection. Furthermore, antibodies against HTLV-1 pass from the maternal blood to the fetal blood freely resulting in acquired passive immunity. During breast feeding the infant acquires HTLV-1 infected cells through the maternal colostrum and usually expresses pathology after infant blood levels of anti-HTLV-1 decline [3]. Transmission of HTLV-1 is mostly dependent on the cell to cell contact with infected cells rather than cell free virions.

Once transmission takes place, HTLV-1 reverse transcribes its RNA genome into DNA and integrates into the cellular DNA becoming provirus. Following integration viral gene expression occurs via host cellular machinery and through replication of integrated provirus. Some of the important genes on the proviral DNA include tax and rex transregulatory genes, which play an important role in replication and cellular

transformation (hijacking). Others such as *gag* encode for a structural polypeptide that is later cleaved by viral protease into matrix, capsid and nucleocapsid proteins. The *pol* gene encodes for the reverse transcriptase and other enzymes such viral integrase. Most importantly however, as a vaccine and therapeutic target, is the viral envelope that is responsible for the attachment and penetration of the virus. It is encoded by the *env* gene and is expressed on the surface of infected cells [3].

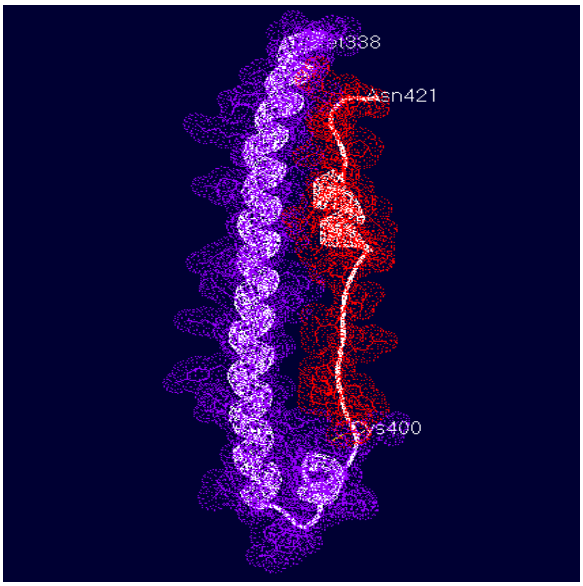
The initial product of the env gene is a glycosylated protein gp62 made up of 488 amino acid residues [4]. It is cleaved in the Golgi apparatus into two final subunits, gp46 and gp21. Gp46 surface subunit (SU) consists of residues 1-312. The gp21 consists of amino acid residues 313-488 and is referred to as the transmembranous (TM) region which spans the lipid bilayer membrane of the infected cell. Both subunits interact via noncovalent intermolecular forces and together they mediate receptor specific binding and fusion of viral or HTLV-1 infected cellular membrane with the membrane of the host cell resulting in entry of the virus or the formation of gigantic multinucleated cells called syncitia .

Previous research has been conducted for exploring the important regions within gp21 and gp46 subunits that are directly involved in binding and fusion processes of HTLV-1 transmission. Sagara et al. reported 23 peptide sequences, covering the whole Env protein, with overlapping residues were synthesized and tested for their ability to inhibit syncitium formation by HTLV-1 infected cells [1]. The inhibition of Syncitia formation by the peptides indicated that the corresponding sequences on the Env protein are crucial for the transmission of the virus. It was determined that residues 400-429 (P400) on gp21 as well 197-216 (P197) on gp46 strongly inhibited syncitium formation

and therefore were important for the function of Env. Additionally, P400 was found to be more profound than the P197 region in the inhibition of syncitium and therefore was predicted to have a more important role in the viral transmission. It was later shown that P400 caused a reduction in infection by cell free HTLV-1 virions whereas P197 displayed no effect in reducing infection [5]. This finding also supports the initial hypothesis that P400 plays a more crucial role in the overall process of HTLV-1 infection.

According to the X-ray crystal structure of gp21, P400 sequence is located between the anchorage domain and the coiled coil region [6]. The anchorage domain corresponds to the hydrophobic sequence 446-465 at the carboxyl terminus of the gp21 subunit and the coiled coil structure corresponds to residues 338-387. There is currently no crystal structure for gp46 but its proposed function is binding and attachment to the putative HTLV-1 receptor [7]. The attachment and entry mechanism of HTLV-1 has been found to resemble HIV. After the initial step of gp46 binding to the receptor, the fusion domain, corresponding to the 29 amino acid hydrophobic stretch at the putative amino terminal part of gp21, inserts into the host cell membrane. This event is termed prefusion state. This step induces conformational changes in the envelope structure where the hinge region folds allowing P400 to interact with coiled coil region of the gp21 TM subunit forming a trimer of hairpins (figure 1). This new conformation is highly fusogenic as it brings the host cellular membrane and infected cell or viral membrane within close proximity. Consequently both membranes fuse together allowing viral entry or syncitium formation [6, 8]. This structural analysis leads to the conclusion that P400 is important for the fusion and post binding stages of HTLV-1 infection. In addition, mutagenesis

Fig1.The crystal structure of gp21 transmembranous subunit in its fusogenic state. The 400-429 sequence (red) interacting with the coiled coil region forming a trimer of hairpins. This interaction is mediated by the hinge region between the coiled coil and 400-429 sequences.


t388
sn421
s400

studies have revealed that Asn407, Ser408, and all leucine residues have an important role in the function of P400 [5].

The role of P197 has been initially hypothesized as being involved in the initial binding of the putative HTLV-1 receptor in the attachment step. This conclusion has been derived from the observation that syncitium formation was inhibited after the *in vitro* addition of LAT-27 monoclonal antibodies, which target residues 191-196, followed by detection of proviral load using PCR assay [5]. However, further studies involving competitive binding experiments using recombinant gp46 protein refuted this initial hypothesis, indicating that it may be involved in post binding steps [9]. But nevertheless, P197 has been determined as crucial for the process of viral infection as inferred from its ability to inhibit syncitium formation [1].

After the appearance of the findings that relate to P400 and P197 sequences and their importance in viral fusion, there has been an emergence of a new field of research that involves investigating the possibility for the development of HTLV-1 fusion inhibitors as a therapeutic strategy against HTLV-1. This has been encouraged by the success achieved in the development of fusion inhibitors as therapeutics against HIV. In HIV, the envelope contains two subunits similar to HTLV-1: the gp120 surface subunit, which resembles gp46 in HTLV-1, and the gp41 transmembranous subunit, which resembles gp21 in HTLV-1 [10]. The gp41 subunit contains two heptad repeats. One lies close to the amino terminus (HR1) and the other lies close to the carboxyl terminus (HR2). At the fusogenic state both heptad repeat regions interact together noncovalently forming a trimer of hairpins. A potent viral fusion inhibitor (T-20 or Fuzeon) has been

successfully made, which mimics the HR2 portion of the gp41. The presence of this peptide hinders the formation of the trimer of hairpin structure by interacting with the HR1 domain instead of the original carboxy terminal HR2. Consequently the fusogenic state is not achieved and no infection can occur [10].

The success of T20 has inspired efforts into the development of peptide fusion inhibitors against HTLV-1 using synthetic peptides that are designed to mimic regions on HTLV-1 Env that are crucial for viral fusion. Also the fact that HTLV-1 Env sequence is highly conserved as compared to HIV Env, suggests that the development of therapeutics against HTLV-1 is more attainable than HIV. P400 and P197 sequences have been the first documented designs of fusion inhibitors since they were found to inhibit syncitia formation and therefore prevent viral transmission. P400 interacts with the coiled coil groove preventing the natural sequence from binding and forming a trimer of hairpins structure. Consequently fusion is inhibited in a similar manner to that of HIV. P197 interacts with the gp46 in a post binding step of unknown mechanism preventing fusion in a less potent manner than P400 [9]. Another attempt in the design of HTLV-1 viral fusion inhibitors was the design of a P400 mutant ($P^{cr}400$), which is naturally expressed in the CR strain of HTLV-1. The regular P400 sequence is expressed in ATK HTLV-1 strain. $P^{cr}400$ was shown to be more effective in inhibiting syncitia than P400 [11].

Recent developments in HIV therapeutic strategies increased the importance of the investigation into the possibility of producing longer half life treatment. This is needed to overcome the disadvantages faced by peptide therapeutics such as low bioavailability and high frequency of dosage. Peptide therapeutics are prone to degradation by body proteases and therefore they have a shorter half life. As observed

with T-20 the dosage frequency required to reduce the viral load in patients' blood is high (2 mg/kg body weight twice a day) and therefore makes it more expensive to use in the treatment of AIDS [10]. As a result new strategies in the improvement of the half life could reduce the frequency of therapeutic doses per day reducing the expense of and increasing the practicality of the treatment.

One such strategy involves the use of retro-inverso peptides to bypass the protease susceptibility problem [12]. The usage of D-amino acid isomers instead of the original L-amino acids makes the peptide protease resistant since D-amino acids are not natural and therefore body proteases do not have specificity for it. In spite of that fact, using D-amino acids with the same peptide sequence would result in a different optical peptide isomer which may function in a different manner than the parent peptide. Therefore the use of D-amino acids only is not sufficient to produce the desired peptide with protease resistance. In addition to that the peptide backbone sequence should be inverted to yield a peptide with inverted sequence and inverted amino acid chirality. The result of both changes is a peptide with almost identical overall structure and chemical topology as the original L-amino acid peptide. Each change separately would produce a totally different peptide that loses its desired function. This effect is called the retro-inverso effect and its implementation in the peptide therapeutic development would improve the half life and retain the similar desired function as expected. The main drawback of this strategy is that it is very expensive due to the use of D-amino acids. Therefore it requires careful consideration and planning before its implementation.

This project pursues two main goals. Since P400 was previously found to be more effective than P197 using *in vitro* analysis, it will be the first to be evaluated for the

possibility of developing a retro-inverso (RI) version. Then based on the efficacy of the RI peptide in comparison with its parent peptide, P400, an investigation into the possibility and practicality of developing a retro-inverso version of P197 will be considered. In both cases the idea is to carefully plan before synthesizing the more expensive retro-inverso version of the peptide, by experimentally determining the benefits and costs of the decision. If the observed benefits exceed the costs then RI peptides would be synthesized and evaluated for their efficacy in achieving the desired therapeutic goals.

To accomplish the first goal, P400 and $P^{cr}400$ were evaluated *in vitro* for their comparative efficacy since both correspond to the same region on gp21. A three cell line syncitia inhibition assay, involving β galactosidase chemiluminescence detection, was utilized for the evaluation process. The more effective of the two peptides in inhibiting fusion, was found to be P400 and therefore was chosen for retro-inverso synthesis. Next, the retro-inverso peptide (RIP400) was tested using the same technique and compared in its efficacy with its parent peptide. The prediction was that the RI peptide should possess similar, although not identical, inhibition properties to its parent peptide. In this case retro-inverso strategy would have displayed a high degree of success. However, RIP400 was observed to have less efficacy than P400 as a fusion inhibitor. Further investigations were carried out to find out possible reasons behind this result. Additionally, RIP400 was found to be similarly effective to P197 in inhibiting syncitia and therefore was considered as a legitimate potential therapeutic because P197 is a documented relatively effective fusion inhibitor.

In order to accomplish the second goal, P197 was evaluated with 175-218 peptide sequence for the possibility of finding a better alternative. 175-218 is a sequence on the gp46 subunit of Env and was found to be a potent B cell epitope in eliciting high levels of anti-Env antibodies [13]. This sequence encompasses the P197 sequence and would therefore be tested for its potency in inhibiting syncitia. If 175-218 demonstrated a higher efficacy than P197, it might then be evaluated further for retro-inverso strategy instead of P197. It was observed however, that both P197 and 175-218 displayed similar efficacy levels in fusion inhibition, therefore 175-218 was not further considered for HTLV-1 therapeutic development. In addition to that, P197 was evaluated as a 1:1 mixture with P400 in order to determine if there is any added advantage of a combined effect or synergism between both peptides. Since both peptides mimic different areas of Env it is predicted that both should display synergism. In this case it may be worth synthesizing RIP197 as combination with the gp21 RI peptide synthesized earlier. However, contrary to the synergism hypothesis, the efficacy level of both peptides combined was found to be lower than the efficacy of P400 by itself. Therefore there is no potential added benefit of synthesizing RIP197 to use in conjunction with P400.

The ultimate goal of this project is the design and synthesis of potential therapeutics against HTLV-1 infection, which possess longer half life than available alternatives and yet retain a high degree of efficacy in viral fusion inhibition.

Materials and Methods

Peptide Synthesis: Peptide synthesis was performed on a Milligen/Biosearch 9600 solid-phase peptide synthesizer (Bedford, MA) using Fmoc/t-But chemistry. In order to get the amidated C-terminus of the all the peptides, CLEAR amide resin (0.32 mmol/gm) was used for the syntheses (Peptides Interanational, Louisville, KY). After the synthesis of desired length of the peptide, the Fmoc-group from the N-terminus was removed and the completion of the reaction was confirmed by Kaiser test (positive), then it was acetylated on resin using N-acetyl-imidazole in DMF (4h), completion of the acetylation was confirmed by the Kaiser test (negative). All peptides were cleaved from the resin using the cleavage reagent B (Trifluoroaceteic acid:Phenol:Water:Triisopropylsilane 90:4:4:2) and crude peptides were purified on RP-HPLC as reported earlier [14]. All peptides were characterized by Matrix Assisted Laser Desorption Ionization mass spectroscopy (MALDI) (Campus Chemical Instrumentation Center, The Ohio State University, Columbus, Ohio)(Table 1).

Circular Dichroism: A 100 μM peptide concentration was prepared using water in one trial and 50 % trifluoroethanol (TFE) in another trial. Then spectral measurements were obtained for each of the peptide solutions in a quartz cuvette 0.1 cm path length, at 25 °C and under continuous nitrogen purging of the sample compartment. Readings were measured every 1 nm over a range of wavelengths (260 nm down to 190 nm). 5 s averaging time was set between readings. The instrument utilized was an AVIV model 62A DS CD (Lakewood, NJ). The measurements were expressed in millidegrees and were subtracted from water and 50 % TFE blank readings.

Table1. All the peptides synthesized and utilized in this project.

Peptide ID	Sequence	Number of amino acids	Molecular weight (g/mol)
P400	Ac-CRFPNITNSHVPILQERPPL ENRVLTGWGL-NH2	30	3498
Pcr 400	Ac-CCFLNITNSHVSILQERPPL ENRVLTGWGL-NH2	30	3450
RIP400	Ac-LGWGTLVRNELPPREQLI PVHSNTINPFRC-NH2	30	3498
	D-Amino Acids		
P197	Ac-DHILEPSIPWKSKLLTLV QL-NH2	20	2371
175-218	FLNTEPSQLPPTAPPLLPHSNLDHILEPSIPWKSKLLTLVQLT	43	4707
SCRD40L	Ac-PSEIVQRPIFGLWKSLRSGEPILS-NH2	24	2749
STEP CD28	Ac-KIPTGLNPRNSMEYEDFPIY-NH2	20	2425

Mean residue ellipticity (θ) was calculated using the following formula: θ = millidegrees x M_w / 10 x c x l x n, where M_w is the molecular weight of peptide, c is the concentration of peptide used in mg / ml, n is the number of amino acids, and l is the path length [15]. Additionally, percentage helicity at 222 nm was calculated according to Chen's equation [16] with reference to the mean residue ellipticity at 222 nm for 100 % α-helix is = -35,700. 100% helicity for a particular peptide at 222 nm (Xh^n) is calculated using -35,700 / (1-K / n), where k is a constant that = 2.57, and n is the number of amino acids in the chain. Then the calculated value of θ at 222 nm of each peptide at each concentration in both solvents was divided by the Xh^n value of the corresponding peptide.

Cell lines: HTLV-1 infected T cell line MT-2 was cultured in RPMI 1640 with 10 % fetal bovine serum (FBS), 1 % penicillin/streptomycin, and 2 mM glutamine. HeLa-Tat cells containing the human immunodeficiency virus (HIV) transcriptional activator Tat gene were cultivated in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum, and 1 % penicillin/streptomycin. CosZ28 cells containing β-galactosidase gene under regulation by HIV long terminal repeat promoter, were grown in same medium as HeLa-Tat cells with 300 µg/ml of hygromycin as a selective agent. Cos7 cells were cultivated in the same culture medium as HeLa-Tat cells.

Syncitia Inhibition Assay: The protocol employed was a modification from Sundaram et al [14]. 5 x 10^4 MT-2 cells per well were incubated in 400 µl of MT-2 culture medium with various peptide dilutions in 24-well plates for 1 h at 37 °C in 5 % CO_2. The peptides were prepared by dissolving in deionized distilled water followed by serial dilutions in MT-2 culture medium in order to achieve the required concentrations. Following the serial dilutions each of the peptide dilutions was plated in triplicate as

described previously. At the end of the 1 h incubation period, 5.0 x 10^4 CosZ28 and 5.0 x 10^4 HeLa-Tat cells were added in HeLa-Tat culture medium per well. Each of the three replicate plates included two wells that did not contain peptide, one in which all three cell lines were added. This was used as a positive control for measuring maximum luminescence. The second well did not contain MT-2 cells and was used as negative control for detecting background luminescence produced by CosZ28 and HeLa-Tat cells. Following the addition they were incubated for 20 h at 37 °C in 5 % CO_2 after which β-galactosidase levels were measured using the Galactolight Plus kit (Tropix) [17]. 100 µl of lysis buffer was used per well for cell lysis and harvesting. The cell lysate was then added in triplicate at 20 µl per well to 96-well chemiluminescent detection plates (Costar). Then they were incubated for 30 min with 70 µl of a 1:100 dilution of Galacton Plus substrate in reaction buffer diluent as prescribed by the manufacturer. Luminescence was then measured using Veritas microplate luminometer version 1.4 (Turner Biosystems). After the injection of 100 µl per well of accelerator, luminescence was measured for 10 s per well with a 2 s delay between wells. The percentage of Syncitia inhibition at a specific peptide concentration is defined as: (Maximum luminescence without background luminescence – luminescence in the presence of peptide at a specific concentration without background luminescence) / (Maximum luminescence without background luminescence) x 100. All data represent the average of the triplicate trials.

Cross-Reactivity Elisa: Direct Elisa was performed with slight modifications from what has been described previously [18]. Peptides were dissolved in 1x PBS (phosphate buffered saline) to achieve a concentration of 2 µg/ml which were then used to coat 96 well microtiter plates at 100 µl/well. The plates were incubated at 4 °C for overnight.

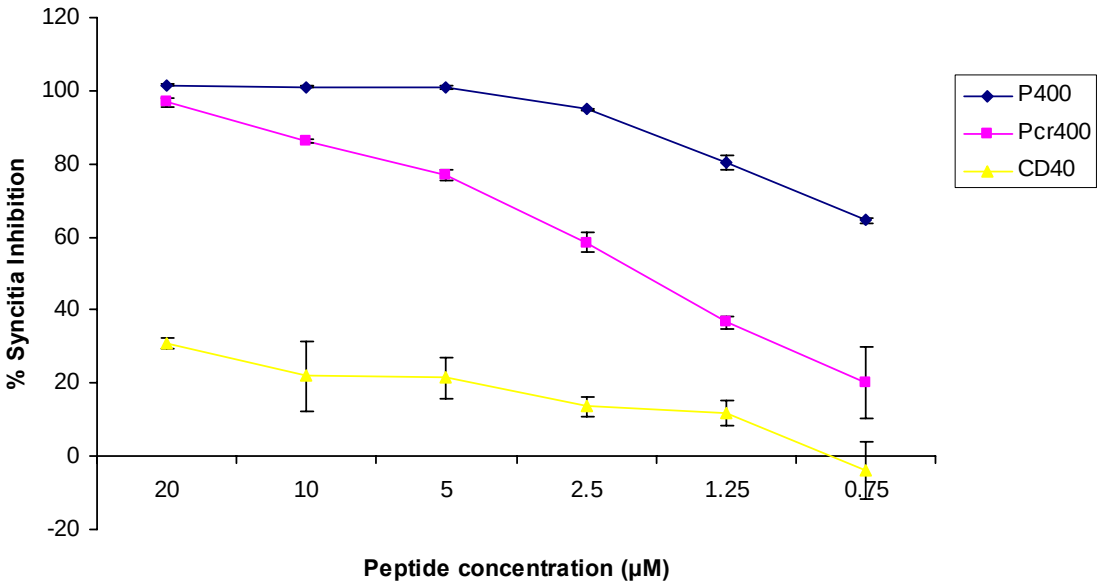
Afterwards the plates were washed four times using wash buffer (PBS-Tween 20 0.05 %, and 1 % horse serum) followed by the addition of 200 µl/well of PBS/1 % BSA containing 0.02 % azide. The plates were then incubated at room temperature for 1 h to allow blocking. The sera of 2 HTLV-1 positive asymptomatic individuals were diluted in wash buffer. Following blocking, the plates were washed and the diluted sera was added in duplicate to the plates at 200 µl per well. Then 2-fold serial dilutions of the sera were performed in wash buffer and the plates were incubated for 2 h at room temperature. At the end of the incubation period the plates were washed and then incubate with 100 µl of a 1:500 dilution of goat anti-human IgG conjugated to horseradish peroxidase (Pierce) as a secondary antibody. After a 1 h incubation period at room temperature the plates were washed with wash buffer followed by tap water and then 50 µl of substrate solution (0.15 %H2O2 in 24 mM citric acid, 5mM sodium phosphate buffer, pH 5.2, with 0.5 mg/ml 2,2'-aminobis(3-ethylbenzthiazoline-6-sulfonic acid) as the chromophore) was added per well. The reaction was allowed to take place for 10 min after which it was stopped using 25 µl per well of 1 % SDS. Absorbance was measured in all wells at 415 nm using a Benchmark microplate reader (Bio-Rad, Hercules, CA). Background absorbance was also determined and subtracted from the raw results before collation. All data represent the average of the duplicate trials.

Results and Discussion

P400 is a better candidate for retro-inverso than $P^{cr}400$. The first aim was to investigate, which of the available gp21 L-peptides was the best candidate for the first attempt for the retro-inverso strategy. The available peptides were P400 and $P^{cr}400$. It was previously shown that $P^{cr}400$ possesses a superior neutralizing capacity than P400 [11]. For further confirmation both peptides were tested with a negative control peptide SCRCD40L (a scrambled peptide sequence derived from the CD40 ligand protein) in a three cell line Syncitia inhibition assay. In the absence of peptides, the MT-2 cells expressing env bind to the putative HTLV-1 receptors on CosZ28 and HeLa-tat cells then undergo fusion resulting into the formation of Syncitia. Fusion of HeLa-Tat and CosZ28 caused by Env Fusion leads to transcriptional activator from the HeLa-Tat cells activating the expression of β galactosidase in the CosZ28 cells through the HIV LTR promoter. The level of β galactosidase was measured in the absence and presence of peptide dilutions and then syncitia inhibition calculated.

The data was then plotted for the peptide dilutions used, and both P400 and $P^{cr}400$ were compared to each other and to the control (figure 2). Both P400 and $P^{cr}400$ displayed high efficacy in the inhibition of syncitia as indicated by the high curve separation between these peptides and the control peptide (SCRCD40L). SCRCD40L expressed very low levels of inhibition, which was expected from an irrelevant peptide. Both relevant peptides inhibited syncitia in a dose dependant manner as observed by the decline in percentage inhibition at lower peptide concentrations especially below 5 μM. Unexpectedly P400 and $P^{cr}400$ showed neutralizing patterns contrary to previous findings

Fig 2. Syncitia inhibition by P400 in comparison with $P^{cr}400$

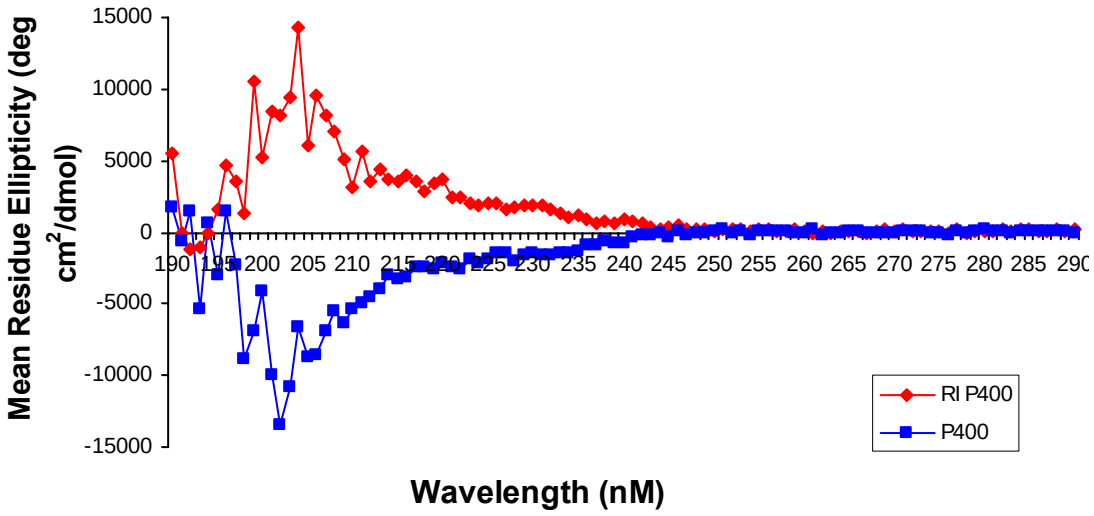

120
100
80
60
40
20
0
-20
% Syncitia Inhibition
20
10
5
2.5
1.25
0.75
Peptide concentration (µM)
P400
Pcr400
CD40

[11]. According to the data obtained, P400 shows a more potent effect as a fusion inhibitor than its mutant $P^{cr}400$. The efficacy of P400 started declining from a 100% inhibition below 5 μM, whereas the efficacy of $P^{cr}400$ started its decline at around 20 μM. One possible explanation of this outcome lies in the structural difference between the $P^{cr}400$ synthesized for this experiment and the $P^{cr}400$ in Pinion et al. The peptide used in this experiment was acetylated at the amino terminus and amidated at the carboxy terminus. The presence of protecting groups at both termini might have interfered with the interaction of the peptide with the coiled coil region of the gp21 region of env resulting in slightly reduced efficacy in inhibiting fusion.

Because P400 was better in the natural form and because of the increased cost of retro-inverso peptides we chose to only make the retro-inverso version pf P400 (RIP400), for future experiments.

RIP400 displays partial efficacy as a fusion inhibitor. The retro-inverso P400 peptide (RIP400) was synthesized as previously described, except that D-amino acids were used instead of the L-amino acids and peptide backbone sequence was inverted to achieve an almost identical side chain topology as the parent peptide (Table 1). The comparison between the circular dichroism measurements of both peptides, P400 and RIP400, confirmed the prediction about the retro-inverso effect (figure 3). Inverting the amino acid sequence of the peptide normally would yield a totally different peptide, but the use of D-amino acid isomers instead of the regular L-amino acids in addition to the sequence inversion, would yield a peptide with the same chemical topology and structure as the parent peptide. The data represents the mean residue ellipticity of the peptides in water over a range of wavelengths (figure 3A). The data chosen represents a 100 μM

Fig 3A. CD measurements of P400 and RIP400 in mean residue ellipticity over a range of wavelengths in water under continuous nitrogen purging.


Mean Residue Ellipticity (deg cm2/dmol)
15000
10000
5000
0
-5000
-10000
-15000
190 195 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290
Wavelength (nM)
RI P400
P400

concentration of both peptides. The peptides were also evaluated in 50 % TFE which favors helicity more than water and therefore shows higher ellipticity values than water (figure 3B). The data shown indicates that both peptides are very similar in overall structure and level of helicity, except that RIP400 shows positive plot and P400 shows negative plot. This is expected since the side chains of RIP400 are D-isomers. One would expect that if the peptide backbone sequence were inverted with out using D side chains, the peptide will loose its similarity to the parent peptide in the CD profile. Since the overall CD trend is very similar in absolute value, the design of RIP400 has been successful.

RIP400 was tested in vitro using the syncitia inhibition assay against P400 and the control peptide SCRCD40L. RIP400 retained partial efficacy from its original (L) version P400 (figure 4). At high concentrations RIP400 was very effective in inhibiting syncitia. 100 % inhibition was achieved at 100 μM. This was followed by a steep decline to 44 % at 10 μM, whereas P400 maintained 100 % inhibition at the same concentration. The results obtained were unexpected because RIP400 resembled P400 in overall structure and topology, as inferred from the CD data. The expectation was that RIP400 should display a similar level of efficacy to P400. A possible explanation for the outcome might lie in the type of interaction between P400 and coiled coil region of gp21. The overall resemblance of structure between P400 and RIP400 does not disregard the differences between them in the type of amino acids used and their sequence in the peptide backbone. Therefore if the interaction of coiled coil region primarily takes place with a certain aspect of P400 rather than the overall structure, then the use of retro-inverso strategy would not be completely successful. For instance, RIP400 would not be

Fig 3B. CD measurements of P400 and RIP400 in mean residue ellipticity over a range of wavelengths in 50% TFE under continuous nitrogen purging.

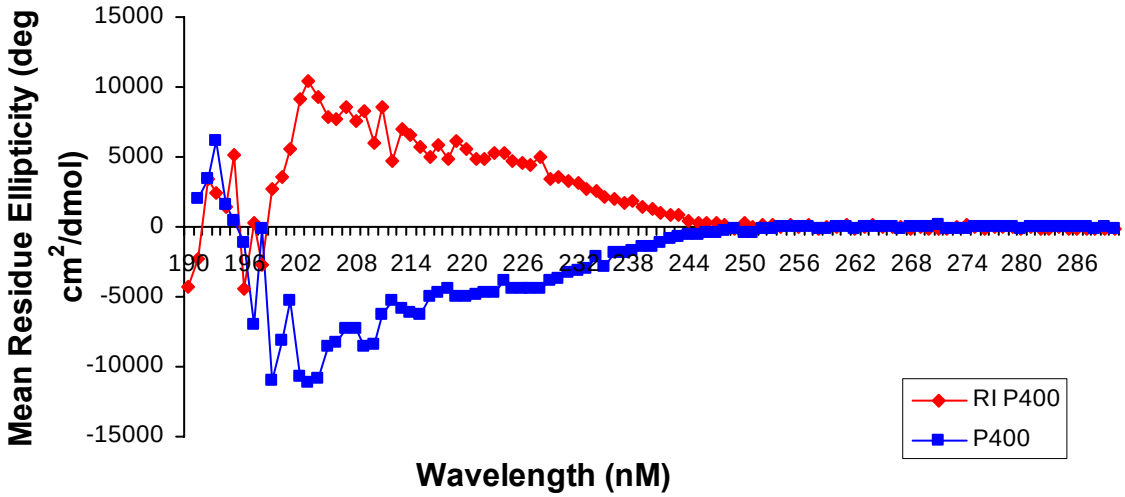
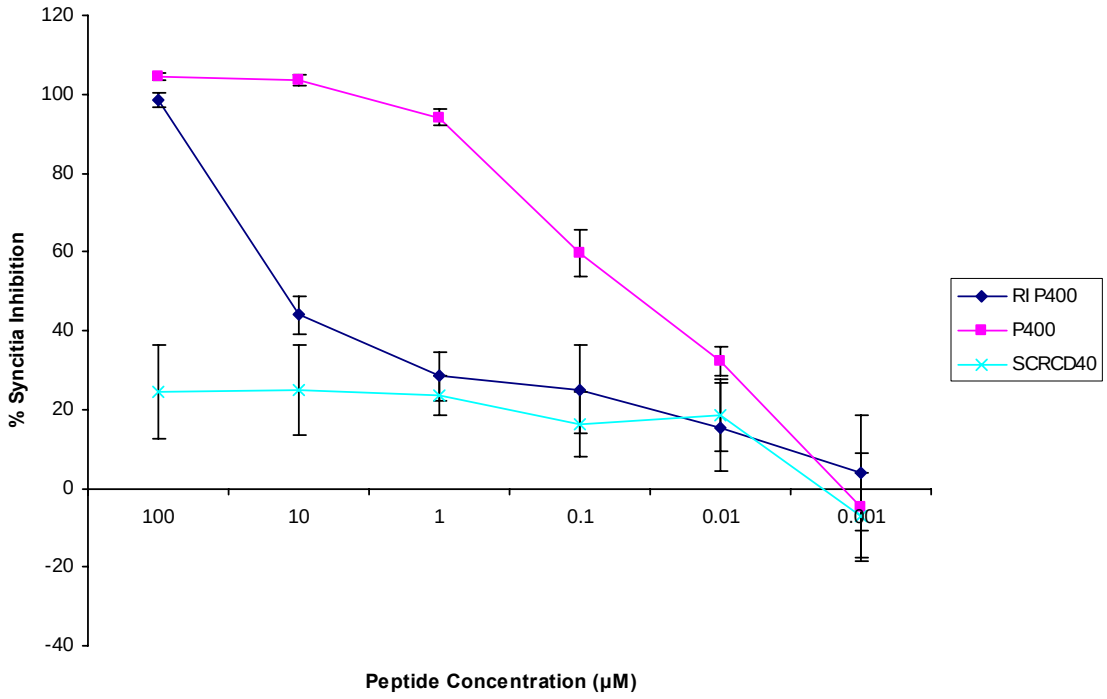

Mean Residue Ellipticity (deg cm2/dmol)
15000
10000
5000
0
-5000
-10000
-15000
190
196
202
208
214
220
226
232
238
244
250
256
262
268
274
280
286
Wavelength (nM)
RI P400
P400

Fig 4. Syncitia inhibition by RIP400 in comparison with P400

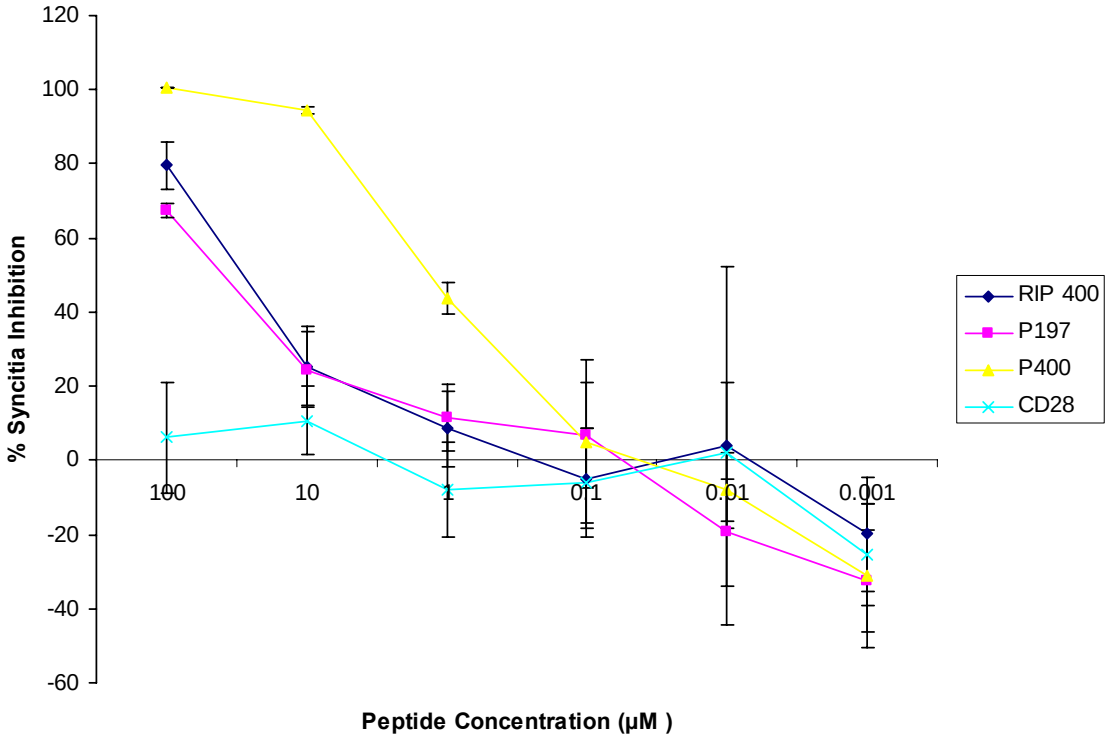

120
100
80
60
40
20
0
-20
-40
% Syncitia Inhibition
100
10
1
0.1
0.01
0.001
Peptide Concentration (µM)
RI P400
P400
SCRCD40

as effective in interacting with the groove in the coiled coil region as P400 due to the D backbone orientation which is slightly different than P400 backbone. Additionally, side chain interactions between the RIP400 and the coiled coil region may be less effective than P400 because D-amino acids might not be as compatible with the coiled coil side chains as L-amino acids in P400 at the site of interaction. Together these reasons pose certain difficulties in achieving a similar efficacy in fusion inhibition to P400.

Nevertheless, RIP400 showed significant inhibition of syncitia especially when compared with the second documented fusion inhibitor P197. Both displayed similar efficacy in inhibiting syncitia at high concentrations (figure 5) P197 and RIP400 displayed similar dose-dependant levels of syncitia inhibition as depicted in the separation between their curves and the control peptide (STEPCD28) above 10 μM. This finding is important in supporting the idea that RIP400 possesses a considerable level of efficacy even though it is not as good as P400. But the similarity with P197 contributes to its legitimacy in terms of how effective it is.

The findings obtained by the syncitia inhibition assay were different from what was predicted through the CD data. The CD data led to the prediction that RIP400 has a structure that is exactly the same as P400 after using D-amino acids and inverting the peptide backbone sequence. Therefore according to the prediction, RIP400 should display similar efficacy to P400 in inhibiting syncitia since both have the same overall structure. Unfortunately, the results were not as predicted. The discrepancy between the results obtained and previous predictions required additional insight into the comparative structures of P400 and RIP400. Therefore cross-reactivity Elisa assay was conducted to determine the comparative level of recognition of RIP400 and P400 by anti-Env

Fig 5. Syncitia inhibition by RIP400 in comparison with P197. P400 was used as a positive control.


120
100
80
60
40
20
0
-20
-40
-60
% Syncitia Inhibition
100
10
1
0.1
0.01
0.001
Peptide Concentration (μM)
RIP 400
P197
P400
CD28

antibodies present in sera of HTLV-1 asymptomatic individuals. Peptides P400 and RIP400 were used in coating the wells and sera of nine patients were used as primary antibodies over a range of concentrations. The primary antibodies should be able to recognize Env peptides such as P400 since they come from HTLV-1 positive individuals. The data indicated that the anti-env antibodies were able to recognize both peptides at varying degrees (figure 6). The detection of RIP400 by anti-Env antibodies suggest that the orientation of RIP400 side chains resembles P400. But in spite of the fact that both peptides were recognized, both did not display similar cross reactivity with all the patients. Each patient serum contains antibodies with different quantities and specificity for different epitopes on the peptides. Therefore some patient sera would show more similar cross reactivity with P400 and RIP400 especially when the antibody quantity in the serum is low (figure 6A). Another patient's sera, for instance patient M, contains a high quantity of antibodies against Env (figure 6B). Therefore there would be a wider variety of antibodies that target different regions within P400. Consequently, greater difference is observed in cross reactivity between P400 and RIP400 because both peptides are not identical. RIP400 has a different peptide backbone sequence as well as D-amino acids as side chains.

Despite the fact that RIP400 did not resemble P400 in potency, it still remains considerably effective at higher dosage level. This must be taken into consideration when deciding the dosage level as compared to its frequency. According to *in vitro* assays it seems that a larger quantity of RIP400 is needed to induce the same effect as P400, but this may be more favorable and less expensive than the higher frequency of dosage that would be required if P400 would be used as opposed to RIP400. Therefore, future *in vivo*

Fig 6A. Cross-reactivity elisa using anti-env antibodies from Patient 1 sera against P400 and RIP400

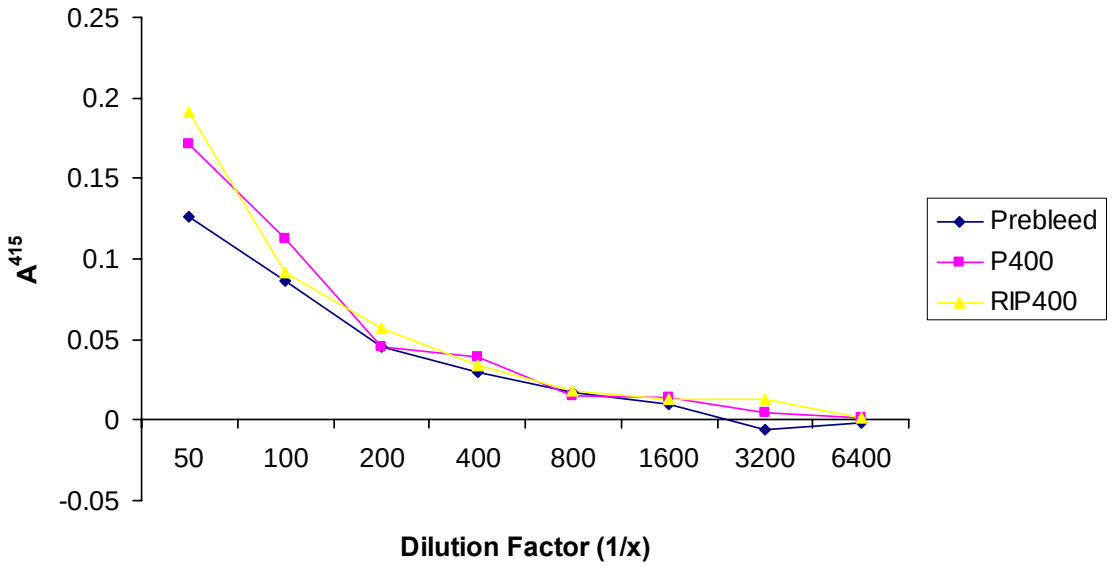
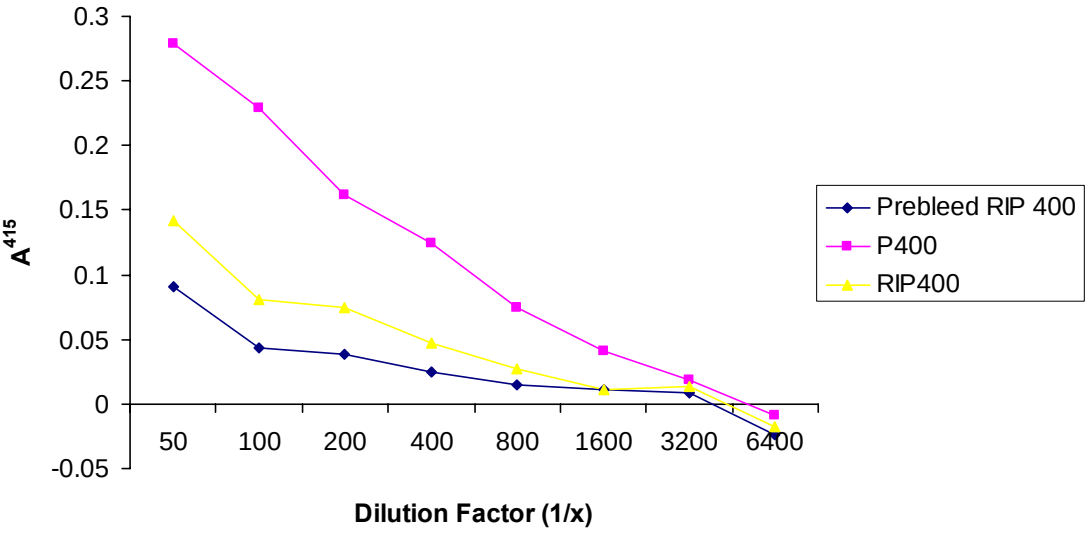

0.25
0.2
0.15
0.1
0.05
0
-0.05
A415
50
100
200
400
800
1600
3200
6400
Dilution Factor (1/x)
Prebleed
P400
RIP400

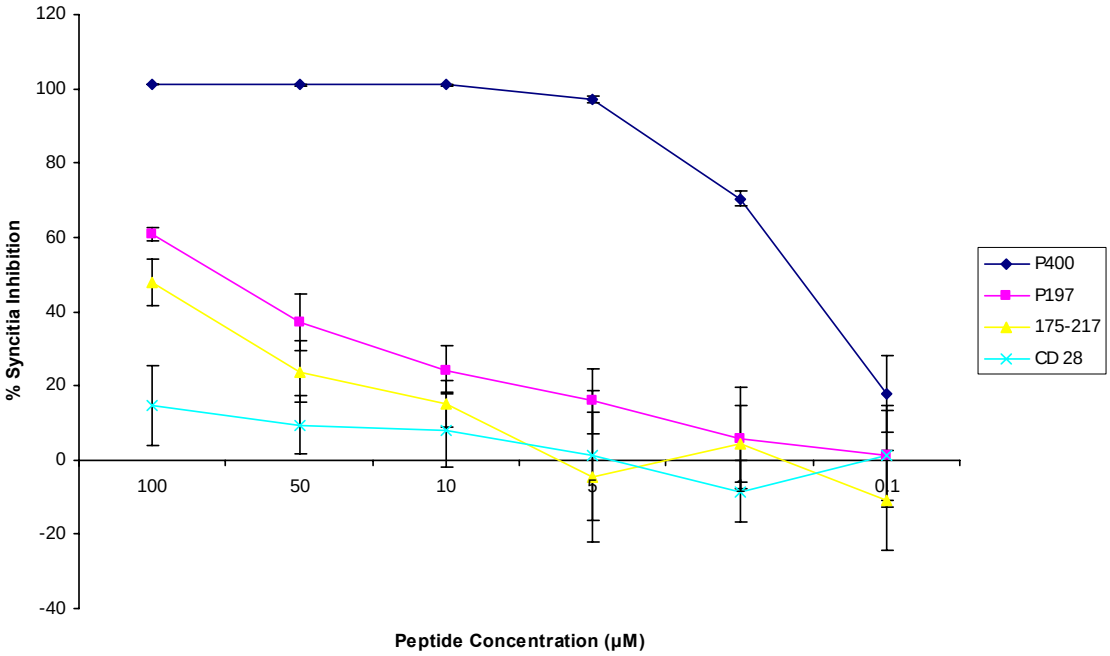
Fig 6B. Cross-reactivity elisa using anti-env antibodies from Patient M sera against P400 and RIP400


0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
A415
50
100
200
400
800
1600
3200
6400
Dilution Factor (1/x)
Prebleed RIP 400
P400
RIP400

experiments are necessary for cost benefit analysis between the two peptides. Conclusively, RIP400 possesses a considerable level of efficacy at higher concentration.

P197 is not a preferable candidate for retro-inverso strategy. The second alternative fusion inhibitor P197 was tested for the retro-inverso development possibility. Since only two main sequences were discovered on HTLV-1 env that inhibited viral fusion, there are limited possibilities that are worth investigating for improvement. P197 which corresponds to 197-216 residues on the gp46 surface subunit was previously proven to be effective to certain extent, even though less effective than P400, in the inhibition of viral fusion [1]. The results obtained confirmed this fact (figures 5, 7). There were two main approaches that were pursued in order to determine whether P197 was a potential candidate for synthesizing its more expensive version RIP197. The first was to test 175-218 peptide sequence of gp46 subunit as an alternative to P197 in order to search for an improved version of P197. 175-218 was previously used as a B cell epitope in HTLV-1 vaccine research and it proved to be very effective in eliciting an anti-Env antibody response. The main idea behind this approach was to explore a more effective alternative to P197 in viral fusion inhibition by the inclusion of 175-196 residues. If 175-218 peptide demonstrated a greater efficacy in the inhibition of syncitia that it would be considered as a better candidate than P197 for retro-inverso strategy. The second approach involved testing the P197 in a 1:1 mixture with P400 and comparing the efficacy of the mixture as opposed to each peptide individually. The prediction was that since P400 and P197 target different areas of HTLV-1 env, then there might be a possibility that they would work synergistically and produce a more effective combination that inhibits viral fusion. If such result is achieved then it would be important to take into consideration the

Fig 7. Syncitia inhibition by P197 in comparison with 175-218

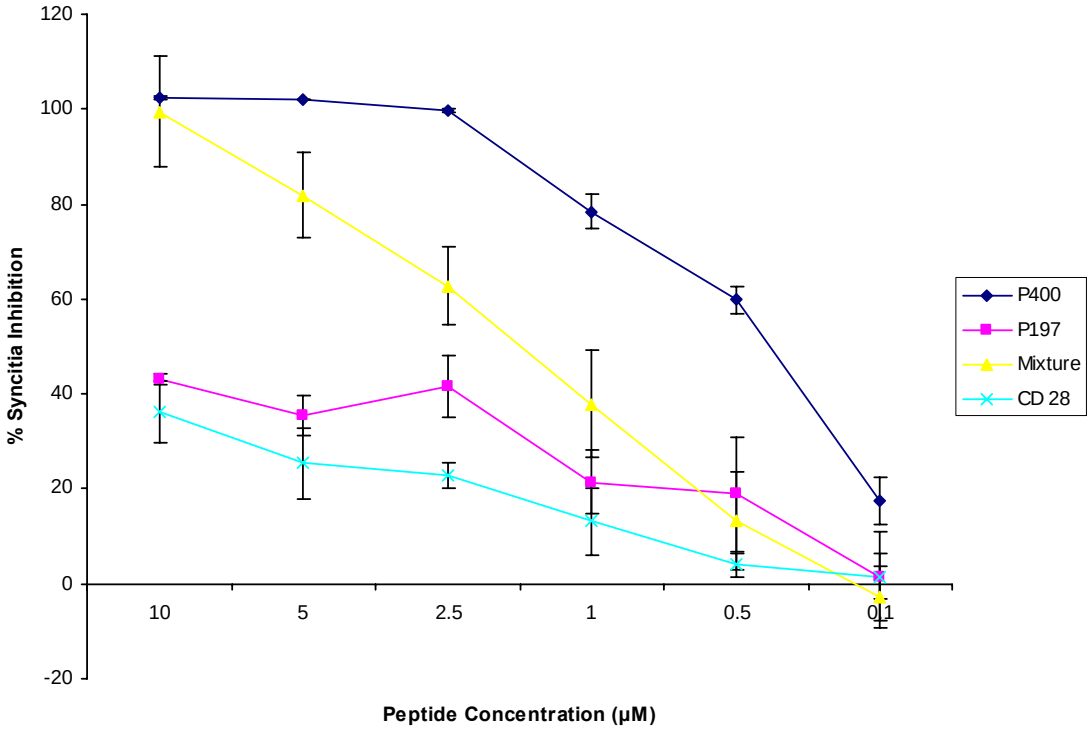

120
100
80
60
40
20
0
-20
-40
% Syncitia Inhibition
100
50
10
5
0.1
Peptide Concentration (µM)
P400
P197
175-217
CD 28

possibility for developing a retro-inverso P197 for use in combination with RIP400 as a longer half-life and effective therapeutic strategy against HTLV-1.

According to the results of the syncitia inhibition assay where P197 was tested with B cell epitope 175-218, both peptides behave similarly in inhibiting the formation of syncitia with relatively significant efficacy above 50 μM (figure 7). This finding indicates that 175-218 peptide does not show improvement compared to P197. This data correlates with the findings of Sagara et al. where among the overlapping peptide sequences tested, 175-196 did not show detectable levels of syncitia inhibition. Therefore a peptide that includes this sequence in addition to 197-216 sequence would not result in any improvement and consequently there is no alternative to P197 for retro-inverso strategy in terms of a peptide that targets gp46.

The second approach described previously was then implemented as a final investigation into the benefit of synthesizing RIP197. When a 1:1 mixture of P197 and P400 were compared with each peptide independently it was found that they do not act synergistically as predicted (figure 8). The level of syncitia inhibition induced by the mixture was 100 % at 10 μM and then immediately started declining in a dose dependant fashion. Whereas P400 remained 100 % effective in concentrations as low as 2.5 μM. Maximum syncitia inhibition for P197 was about 40 % at 10 μM. This indicates that the mixture is by far more effective than P197 yet less effective than P400. This observation may indicate that instead of synergistic action the addition of P197 interferes and reduces the efficacy of the interaction of P400 with the coiled coil region consequently lowering its efficacy, thus it is not favorable to include P197 and P400 together as a therapeutic

Fig 8. Syncitia inhibition by 1:1 mixture of P400 and P197 in comparison with P400 and P197.


120
100
80
60
40
20
0
-20
% Syncitia Inhibition
10
5
2.5
1
0.5
0.1
Peptide Concentration (µM)
P400
P197
Mixture
CD 28

strategy. It is therefore highly likely to observe a similar effect if RIP400 and RIP197 were included together.

In conclusion, all the results from the two experimental approaches do not support the investment in synthesizing RIP197. Due to the unavailability of a more potent alternative to P197 as well as the possibility of using it in combination with P400, it would be less likely that RIP197, if synthesized and tested, would show better results than RIP400.

Conclusions. Based on the findings of this project, both P400 and P197 inhibit syncitia in vitro with P400 being more effective than P197. Therefore they are considered as potential fusion inhibitors, which is consistent with what has been determined previously. Additionally, P400 was to shown to have been more effective in fusion inhibition than $P^{cr}400$, contrary to what has been previously discovered, and therefore was a better candidate for retro-inverso strategy. Furthermore, the retro-inverso strategy was partially successful as shown by syncitia inhibition data of RIP400 and P400. RIP400 displayed less efficacy than P400 with fusion inhibition. But nevertheless, RIP400 showed considerable efficacy as a potential therapeutic when compared with previously reported P197.

In addition to investigations conducted with P400, P197 was also investigated for improvement. 175-218 B cell epitope was tested as possible alternative to P197, but according to the data there was no noticeable improvement in efficacy between both peptides. Moreover, P197 was tested as a mixture with P400 for the possibility of synergistic activity in fusion inhibition between both peptides. According to the data slightly diminished efficacy of the mixture as compared to P400 was observed, which

was contrary to what was anticipated. This outcome was attributed to the lower amount of P400 present in the 1:1 mixture as compared to the P400 solution indicating the absence of synergism between P400 and P197. Due to the findings observed with P197 experiments, the pursuit of a more expensive retro-inverso version was not completed. Future plans may include the use of molecular modeling for investigating improvements to both of the currently existing therapeutic possibilities, P400 and P197. Additionally, alternative methods may be explored for prolonging the half life of the potential therapeutics besides retro-inverso strategy. Also *in vivo* experiments may be conducted with HTLV-1 infected animals in order to determine the actual half-life and efficacy in limiting viral infection by the peptides.

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